eNOS G894T polymorphism as a mild predisposing factor for abdominal aortic aneurysm

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Objective: **Abdominal aortic aneurysm (AAA) represents a chronic degenerative condition associated with atherosclerosis. Actually, data from experimental and clinical studies suggest that nitric oxide (NO) is a modulator in maintaining endothelial function and antithrombotic intravascular environment. Reduced vascular NO generation in subjects carrying the rare variants of the *eNOS* gene might predispose to AAA. No information is available about the influence of the *eNOS* gene T-786C, G894T, and 4a/4b polymorphisms in the susceptibility to the disease.**

Methods: **In this study, we evaluated the role of these polymorphisms in the predisposition to AAA and their influence in hypertensive and normotensive patients. We studied 250 consecutive patients with AAA referred to the Unit of Vascular Surgery of the University of Florence compared with 250 truly healthy subjects with a negative history of vascular diseases. All subjects, patients, and controls, underwent duplex scanning examination, and to assess the presence of other atherosclerotic localizations, all patients underwent clinical and instrumental examinations.**

Results: **A significant difference in genotype distribution and allele frequency was observed for *eNOS* G894T but not for T-786C and 4a/4b polymorphisms. At the multivariate analysis after adjustment for traditional vascular risk factors and other atherosclerotic localizations, the *eNOS* 894T variant was significantly associated with AAA, according to dominant and recessive models (dominant model odds ratio [OR]: 2.2, 95% confidence interval [CI]: 1.21-3.93, $P = .007$; recessive model OR: 2.7, 95% CI: 1.42-5.20, $P = .002$). When patients with other atherosclerotic localizations were excluded from the analysis, the 894T variant still remained associated with the predisposition to AAA, according to the models considered (dominant model OR: 2.1, 95%CI: 1.23-3.92, $P = .007$; recessive model OR: 2.8, 95%CI: 1.45-5.24, $P = .002$).**

Conclusions: **The present study showed that the *eNOS* G894T polymorphism is a mild modulator of the predisposition to AAA apart from traditional risk factors, suggesting a genetic influence on the molecular mechanisms responsible for this complex disease. (J Vasc Surg 2005;42:415-9.)**

Abdominal aortic aneurysm (AAA), which is a chronic degenerative condition, is associated with atherosclerosis. Actually, data from experimental[1] and clinical[2] studies suggest that nitric oxide (NO) is a modulator of vascular disease and its physiologic effects are addressed to maintain endothelial function and antithrombotic intravascular environment; NO contributes to vascular tone regulation, inhibition of platelet aggregation,[3] and leukocyte adhesion to vascular endothelium[4] and inhibits smooth muscle cell migration and proliferation.[5] All these actions likely prevent the development of atherosclerotic plaque. Moreover, experimental data from endothelial NO synthase (eNOS) knockout mice model demonstrated that, in the absence of eNOS, the luminal remodeling is impaired and the vessel wall thickness is doubled, suggesting that endothelium-derived NO, in addition to its role as a vasodilator, may have a role in controlling vessel wall geometry.[6]

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Competition of interest: none.

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The multifactorial pathogenesis of AAA includes environmental and genetic factors interacting to produce the AAA phenotype. Gene encoding for *eNOS* has been proposed as a candidate gene for AAA[3] and polymorphic variants in this gene influence the expression and functional activity of the enzyme. In exon 7 of the *eNOS* gene, the G894T polymorphism has been associated with reduced basal NO production.[7] The mechanism by which *eNOS* G894T polymorphism might reduce NO bioavailability has been related to a selective proteolytic cleavage in endothelial cells and vascular tissues. Since these cleaved fragments would be expected to lack NOS activity, this could account for reduced vascular NO generation in subjects carrying the rare variant.[8]

Another polymorphism in the promoter region of the same gene, called T-786C, is responsible for a reduction of more than 50% in the *eNOS* gene promoter activity, as assessed by luciferase reporter gene assay. The -786C rare allele has been described to be associated with a reduced NO production.[9] Finally, in intron 4, a variable nucleotide tandem repeat (VNTR) has been identified. This polymorphism, also called 4a/4b, has been associated with altered plasma NO levels[10] and has been found to be responsible for variations in the genetic control of plasma nitrite and nitrate levels.[11] Since this polymorphism is intronic, it has been proposed that it might act as a marker in linkage disequilibrium with other functional variants in regulatory regions of the gene.[12]

Data from literature reported information about the *eNOS* 4a/4b polymorphism on the clinical course of AAA progression,[3] but no data are available on the role of all three *eNOS* polymorphisms in the predisposition to AAA.

In the present study we evaluated (1) the role of *eNOS* T-786C, G894T, and 4a/4b polymorphisms in the predisposition to AAA and (2) their influence in hypertensive and normotensive patients.

METHODS

Study population

We enrolled 270 consecutive patients with AAA referred to the Unit of Vascular Surgery of the University of Florence. AAA was defined as a focal dilation of the abdominal aorta at least 50% larger than the expected normal diameter, according to the current report standard.[13] Familial (n = 13) and inflammatory (n = 7) AAAs were excluded from the study. Indeed, familial AAA was considered in patients with one or more first-degree family members with AAA mentioned by the patient and/or diagnosed by a physician, whereas inflammatory AAA was diagnosed on the basis of operative appearance (presence of extensive perianeurysmal and retroperitoneal fibrosis and dense adhesions to adjacent abdominal organs). The final study population comprised 250 AAA patients. A group of 250 truly healthy subjects matched for age and gender were recruited from partners or friends of patients and were used as controls. All controls had a negative history of vascular diseases, as evaluated by expert physicians in the physical examination. Patients and controls were whites from central and southern Italy. All subjects gave informed consent, and the study was approved by the local ethics committee. All subjects, patients and controls, underwent duplex scanning examination using an Acuson Sequoia Color Duplex System (Mountain View, Calif) with a multifrequency convex probe, ranging from 5 to 2 MHz. Ultrasound scanning examination was then confirmed in patients by angiography/computed tomography scan. Digital subtraction angiography was performed only in patients with concomitant peripheral arterial disease and in candidate patients for endovascular treatment of AAA when angiography/computed tomography study was not satisfactory to evaluate the feasibility of endovascular treatment. Aortic diameter was measured below the origin of renal arteries in both groups. The subjects were considered to have hypertension if they had been diagnosed according to the guidelines of European Society of Hypertension/European Society of Cardiology[14] or were taking antihypertensive drugs. Dyslipidemia was defined according to the Third Report of the National Cholesterol Education Program.[15]

To assess the presence of other atherosclerotic localizations, all patients underwent clinical and instrumental examinations (electrocardiography, echocardiography, and duplex scanning with color-coded echo flow imaging on carotid arteries and ankle/brachial pressure index evaluation). In patients with symptoms possibly related to ischemic heart disease, further investigations were performed (echocardiography on drug-induced stress testing, myocardial scintigraphy, and coronary angiography). Severe carotid stenosis, confirmed by angiography/computed tomography, was defined according to the North American Symptomatic Carotid Evaluation Trial criteria.[16] Peripheral arterial disease was documented as present if ankle/brachial pressure index was less than 0.9. On the basis of these criteria, 71 (28%) patients had clinical evidence of coronary artery disease (CAD), 25 patients (10%) had cerebrovascular disease (CVD), and 49 (19%) peripheral arterial disease (PAD).

Aneurysm size was measured by means of computed tomography both in patients and in controls.

Genetic analysis. *eNOS* polymorphisms were analyzed after genomic DNA extraction from peripheral blood leukocytes using a QIAmp Blood Kit (QIAGEN, Hilden, Germany).

***eNOS* T-786C polymorphism.** The *eNOS* T-786C polymorphism has been analyzed by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis. PCR reaction was performed using primers: 5′-GTGTACCCCACCTGCATTCT-3′ (sense) and 5′-CCCAGCAAGGATGTAGTGAC-3′ (anti-sense), and DNA (100 ng) was amplified in a final volume of 20 μL at an annealing temperature of 60°C using a DNA thermal cycler. The PCR detection was followed by Turbo Nae restriction endonuclease digestion for 4 hours at 37°C and resolution by electrophoresis on 2% agarose gel.

***eNOS* 4a4b polymorphism.** The *eNOS* 4a4b polymorphism has been analyzed by PCR-amplification. PCR reaction was performed using primers: 5′-CTATGGTAGTGCCTTGGCTGGAGG-3′ (sense) and 5′-ACCGCCCAGGGAACTCCGCT-3′ (anti-sense) and DNA was amplified in a final volume of 20 μL at an annealing temperature of 58°C. The amplified products were analyzed by electrophoresis on a 3.5% agarose gel.

***eNOS* G894T polymorphism.** The *eNOS* G894T polymorphism detection was performed by real-time fluorescence PCR using the Light Cycler instrument (Roche Diagnostics).

PCR and melting curve determination were performed in a final volume of 20 μL with 10 pmol of each primer NOSF (5′-CACTCCCCACAGCTCTGCAT-3′) and NOSR (5′-CAATCCCTTTGGTGCTCACG-3′), and 4 pmol of each probe (anchor probe and sensor probe). The anchor probe (5′-LC Red 640-CCTTCTGCCCCCCGAGCTGGTCC-3′-P) was 5′ labeled with the LC-Red 640 fluorophore and phosphorylated (P) at its 3′ end to prevent probe elongation by the Taq polymerase. The sensor probe (5′-CCCCAGATGATCCCCCAGAACTC-3′ FLU) was labeled with fluorescein. DNA was amplified at an annealing temperature of 62°C.

The typical melting curve pattern is a single melting peak at a temperature of 65.5°C. For the wild type, the plot is a single melting peak at a temperature of 60.5°C. Heterozygous patients show two melting peaks (60.5°C and 65.5°C).[17]

Table I. Demographic characteristics and traditional risk factors for cardiovascular diseases

Variable	*Patients (n = 250)*	*Healthy subjects (n = 250)*	P
Age (y)	72 (50-83)	71 (49-87)	.06
Males, no. (%)	217 (86.8)	216 (86.4)	.9
Females, no. (%)	33 (13.2)	34 (13.6)	.8
Hypertension, no. (%)	185 (74)	48 (19.2)	<.0001
Dyslipidemia, no. (%)	107 (42.8)	36 (14.4)	<.0001
Smoking habit, no. (%)	180 (72)	62 (24.8)	<.0001
Diabetes, no. (%)	12 (4.8)	9 (3.6)	.5
Abdominal aortic diameter (cm)	6.5 ± 1.3	1.7 ± 1.1	<.0001

Median and (range) for age; mean ± SD for abdominal aortic diameter.

Table II. Genotype distribution and allele frequencies of *eNOS* T-786C, G894T, and 4a4b polymorphisms

Genotype	*Allele*	*Patients (n = 250) No. (%)*	*Healthy subjects (n = 250) No. (%)*	P
eNOS -786 CC		46 (18.4)	42 (16.8)	
eNOS -786 TC		115 (46.0)	116 (46.4)	
eNOS -786 TT		89 (35.6)	92 (36.8)	.9*
	-786C	0.41	0.40	.6†
eNOS 894 TT		69 (27.6)	29 (11.6)	
eNOS 894 GT		109 (43.6)	110 (44.0)	
eNOS 894 GG		72 (28.8)	111 (44.4)	<.0001*
	894T	0.49	0.34	<.0001†
eNOS 4a4a		8 (3.2)	7 (2.8)	
eNOS 4a4b		73 (29.2)	68 (27.2)	
eNOS 4b4b		169 (67.6)	175 (70.0)	.8*
	4a	0.18	0.16	.5†

*Genotype distribution.
†Allele frequency.

Table III. Odds ratios associated with *eNOS* polymorphisms for AAA according to different models: univariate and multivariate analyses

	OR	*95% CI*	P
Univariate analysis			
Age	1.02	0.9-1.05	.08
Gender (males vs females)	1.4	0.7-3.04	.4
Hypertension	11.3	7.4-17.1	<.0001
Smoking habit	7.8	5.2-11.6	<.0001
Dyslipidemia	4.5	2.9-6.9	<.0001
Diabetes	0.7	0.3-1.8	.5
eNOS 894T (dominant model)	1.9	1.3-2.8	<.0001
eNOS 894T (recessive model)	2.9	1.8-4.7	<.0001
eNOS -786C (dominant model)	1.05	0.7-1.5	.8
eNOS -786C (recessive model)	1.1	0.7-1.8	.6
eNOS 4a (dominant model)	1.1	1.7-1.6	.6
eNOS 4a (recessive model)	1.2	0.4-3.2	.8
Multivariate analysis*			
Smoking habit	8.6	4.9-15.1	<.0001
Hypertension	10.4	5.9-18.4	<.0001
Dyslipidemia	2.5	1.3-4.7	.007
eNOS 894T (dominant model)	2.2	1.2-3.9	.007
eNOS 894T (recessive model)	2.7	1.4-5.2	.002

OR, Odds ratio; *AAA*, abdominal aortic aneurysm; *CI*, confidence interval.
*Adjusted for age, gender, traditional vascular risk factor, and other atherosclerotic localizations.

Statistical analysis

Statistical analysis was performed by using the Statistical Package of Social Sciences (SPSS, Chicago, Ill) software for Windows (Version 11.5). Data are expressed as median and range. Aortic diameter was expressed as mean ± SD. The χ^2 test was used to test for proportions and deviations of genotype distribution from Hardy-Weinberg equilibrium. *eNOS* T-786C, G894T, and 4a/4b allele frequencies were obtained by direct count. The number of subjects studied was sufficient to detect, with a statistical power of 80% ($\beta = 0.8$) and significance value of .05 (α), absolute difference for *eNOS* G894T polymorphism. The nonparametric Mann-Whitney test was used for comparisons between single groups. Kruskal-Wallis test was used for comparisons among different groups. To assess different models at each locus, genotypes at each position were coded as recessive model (eg, *eNOS*-786CC vs -786TC+TT) and as dominant model (eg, *eNOS*-786CC+TC vs TT). The relationship between the three polymorphisms and AAA was determined by univariate logistic regression analysis. Variables showing, at univariate analysis, a statistically significant association with AAA were introduced in a multivariate model to evaluate the association with the disease after adjustment for traditional cardiovascular risk factors. Odds ratio (OR) with 95% confidence interval (CI) was determined. $P < .05$ was considered to indicate statistical significance.

RESULTS

Demographic and clinical characteristics of the study population are described in Table I. We observed significant differences between the two groups for traditional cardiovascular risk factors. Genotype distribution and allele frequency of *eNOS* T-786C, G894T, and 4a/4b polymorphisms were in Hardy-Weinberg equilibrium and are reported in Table II.

A significant difference in both genotype distribution and allele frequency between patients and healthy subjects was observed for *eNOS* G894T, but not for *eNOS* T-786C and 4a/4b polymorphisms.

The univariate logistic regression analysis showed a significant association of *eNOS* 894T, but not of -786C and 4a alleles and AAA according to dominant and recessive models (*eNOS* 894T dominant model OR: 1.94, 95% CI: 1.3−2.8, $P < .0001$ and recessive model OR: 2.91, 95% CI: 1.8−4.7, $P < .0001$) (Table III). After adjustment for age, gender, smoking habit, hypertension, dyslipidemia, and other atherosclerotic localizations (Table III), the *eNOS* 894T variant was found to be significantly associated with AAA, according to the models considered. The concomitant presence of *eNOS* 894T rare allele and the other

two rare variants (-786C and 4a) did not affect the predisposition to AAA (*eNOS* 894T and -786C vs 894G and -786T OR: 1.39, 95% CI: 0.5−3.8, $P = .5$; *eNOS* 894T and 4a vs. 894G and 4b OR: 0.65, 95% CI: 0.01−0.9, $P = .9$). When patients with other atherosclerotic localizations (ie, CAD, PAD, and CVD) were excluded from the analysis, the *eNOS* 894T allele still remained significantly associated with a predisposition to AAA, according to the models considered (*eNOS* 894T dominant model OR: 2.2, 95% CI: 1.2−3.9, $P = .007$ and OR 894T recessive model OR: 2.8, 95% CI: 1.4−5.2, $P = .002$).

DISCUSSION

The new finding of this study is the evidence that the *eNOS* gene mildly influences the predisposition to AAA and, in particular, that the G894T polymorphism is mildly associated with the risk for AAA in patients in whom other atherosclerotic localizations, such as CAD, CVD, and PAD, were excluded. Moreover, this study is novel in evaluating, through the analysis of other two polymorphisms, the G894T in the exon 7 and T-786C in the promoter region, both of which are proposed to be functionally involved in the transcriptional pathway, the possible influence of the *eNOS* gene in the pathogenesis of AAA.

NO modulates vascular disease and maintains endothelial function and antithrombotic intravascular environment. Its actions are together likely to prevent the development of atherosclerotic plaques. There is good evidence that NO generation is depressed in blood vessels affected by atherosclerosis as well as in blood vessels exposed to atherosclerotic risk factors. In addition to the acquired defects in NO generation, which can contribute to endothelial dysfunction and to the development of atherosclerosis, there is evidence that inherited differences in endothelial function could also play a role.

eNOS expressed in the endothelial layer is strategically positioned to affect luminal and abluminal processes. An experimental study in a murine model[2] demonstrated that the eNOS deficiency is able to change the disease pattern of atherosclerosis, thus determining PAD, myocardial ischemia, and vascular complications, such as aortic dissection and AAA formation. Moreover, data from eNOS knockout mice provide evidence that NO is a major regulator of vessel reorganization in response to a remodeling stimulus and suggests that a primary defect in NOS/NO pathway can promote abnormal remodeling and may facilitate pathologic changes in vessel wall morphology.[6] Polymorphisms in the *eNOS* gene have been related to impaired expression and functional activity of the enzyme, so modulating the NO availability.

The present study demonstrated that the *eNOS* G894T polymorphism, which has been reported to be associated with coronary artery disease[18] and carotid atherosclerosis,[19] represents a susceptibility to AAA factor. Moreover, our findings, by analyzing the role of all three *eNOS* gene polymorphisms in AAA patients, provided an extended evaluation of the genetic influence of the *eNOS* gene in modulating atherosclerotic predisposition. A number of specific factors can alter the production of NO, thus influencing the progression of atherosclerosis. As conditions when vascular tissue levels of tetrahydrobiopterin (BH4), a cofactor for NOS, are deficient or lacking, eNOS becomes dysfunctional and produces superoxide rather than NO[20]; moreover, in animal model of hyperlipidemia, it has been demonstrated that atherogenesis may be promoted via increased superoxide generation from dysfunctional eNOS.[21]

Actually, the G894T polymorphism might impair the function of eNOS, thereby accounting for the reduction in NO availability.[7] This might be due to the fact that the 894T rare variant, but not the 894G wild-type allele, is susceptible to inactivation by cleavage, possibly because of a tighter turn of the alpha helix,[8] thus determining a reduction in the capacity for NO production.

The study has some limitations: First, because of its case-control design, the study was able to determine the predisposition to the disease for genetic polymorphisms; second, we were not able to perform a Doppler examination in all the relatives, thus excluding familial AAA only on the basis of self-reported data, as confirmed by the physicians. Third, the control group consists of truly healthy subjects with statistically significant differences for traditional risk factors as compared to AAA patients. A population study of patients with AAA compared to subjects without AAA but with similar prevalence of traditional risk factor would be the best way to test the potential association between genetic polymorphisms and the disease.

Our findings demonstrate that the 894T rare variant affects the predisposition to AAA; the effect of one gene on complex diseases, such as AAA, might be difficult to explore in a population study exposed to environmental confounding factors known to influence the disease. To date, hypertension represents a relevant factor affecting the AAA pathogenesis; no definitive data on the role of the *eNOS* gene in modulating the predisposition to hypertension[22,23] are available in literature. Nevertheless, we demonstrated that the effect of the *eNOS* gene is relevant independently from hypertension.

In conclusion, the present study showed that the *eNOS* G894T polymorphism is a mild modulator of the predisposition to AAA apart from traditional risk factors, suggesting a genetically determined influence on the molecular mechanisms responsible for this complex disease and a major direct effect on eNOS activity.

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